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Natural and Genetically Engineered Proteins for Tissue Engineering

Sílvia Gomes[1,2,3], **Isabel B. Leonor**[1,2], **João F. Mano**[1,2], **Rui L. Reis**[1,2], and **David L. Kaplan**[3]

[1]3B's Research Group - Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal

[2]IBB - Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal

[3]Departments of Biomedical Engineering, Chemistry and Physics, Tufts University, Medford, Massachusetts 02155 USA

Abstract

To overcome the limitations of traditionally used autografts, allografts and, to a lesser extent, synthetic materials, there is the need to develop a new generation of scaffolds with adequate mechanical and structural support, control of cell attachment, migration, proliferation and differentiation and with bio-resorbable features. This suite of properties would allow the body to heal itself at the same rate as implant degradation. Genetic engineering offers a route to this level of control of biomaterial systems. The possibility of expressing biological components in nature and to modify or bioengineer them further, offers a path towards multifunctional biomaterial systems. This includes opportunities to generate new protein sequences, new self-assembling peptides or fusions of different bioactive domains or protein motifs. New protein sequences with tunable properties can be generated that can be used as new biomaterials. In this review we address some of the most frequently used proteins for tissue engineering and biomedical applications and describe the techniques most commonly used to functionalize protein-based biomaterials by combining them with bioactive molecules to enhance biological performance. We also highlight the use of genetic engineering, for protein heterologous expression and the synthesis of new protein-based biopolymers, focusing the advantages of these functionalized biopolymers

Correspondence to: David L. Kaplan.

Sílvia Gomes: 3B's Research Group, Department of Polymer Engineering, University of Minho, AvePark, Zona Industrial da Gandra, S. Claudio do Barco, 4806–909 Caldas das Taipas, Guimarães, Portugal. Tel: +351 253 510 900 Fax: +351 253 510 909. silvia.gomes@dep.uminho.pt
Isabel B. Leonor: 3B's Research Group, Department of Polymer Engineering, University of Minho, AvePark, Zona Industrial da Gandra, S. Claudio do Barco, 4806–909 Caldas das Taipas, Guimarães, Portugal. Tel: +351 253 510 900 Fax: +351 253 510 909. belinha@dep.uminho.pt
João F. Mano: 3B's Research Group, Department of Polymer Engineering, University of Minho, AvePark, Zona Industrial da Gandra, S. Claudio do Barco, 4806–909 Caldas das Taipas, Guimarães, Portugal. Tel: +351 253 510 900 Fax: +351 253 510 909. jmano@dep.uminho.pt
Rui L. Reis: 3B's Research Group, Department of Polymer Engineering, University of Minho, AvePark, Zona Industrial da Gandra, S. Claudio do Barco, 4806–909 Caldas das Taipas, Guimarães, Portugal. Tel: +351 253 510 900 Fax: +351 253 510 909. rgreis@dep.uminho.pt
David L. Kaplan: Departments of Biomedical Engineering, Chemistry and Physics, 4 Colby, Street, Rm. 251, Tufts University, Medford, MA 02155. Tel: 617-627-3251 Fax: 617-627-3231. David.Kaplan@tufts.edu

when compared with their counterparts extracted directly from nature and modified by techniques such as physical adsorption or chemical modification.

Keywords

Biomaterials; tissue engineering; natural polymers; recombinant technology; chimeric proteins

1. Introduction

Treatment of injured tissues or organs focuses on the use of autologous and allogenic grafts [1]. However, this practice has significant limitations for the patient and health systems worldwide. Autologous grafts cause donor site morbidity and consequent loss of organ functionality. Allografts are associated with risk of disease transmission and require the use of immunosuppressants with associated side effects [2–4]. In the field of orthopaedic implants, autologous and allogenic grafts account for 90% of the grafts currently used, with synthetic materials (metals, polymers, ceramics and composite systems) used in 10% of surgery cases [3, 5, 6]. In the three types of grafts there are numerous cases of implant failure as a consequence of undesirable local tissue responses resulting in implant loosening, insufficient osseointegration, osteolysis, inflammation and infection [2–4]. These complications account for a failure rate of 13 to 30% in the case of autografts and 20 to 40% for allografts [2].Besides autologous and allogenic grafts, synthetic materials have also been used for controlled drug delivery systems, scaffolds design and orthopaedic fixation as screws, pins or rods [7, 8]. Nevertheless, most synthetic polymers are too hydrophobic and need additional bulk or surface modifications to render the material more biocompatible and suitable for implantation [9]. Therefore, there is a need for alternatives to these practices. Tissue engineering and regenerative medicine offer an approach to circumvent the present therapies with new methods of health care treatment with the purpose of improving the quality of life [2, 10]. This improvement can come in the form of new cytocompatible and non-toxic biomaterials for the manufacture of a new generation of scaffolds comprising adequate mechanical and structural support and able to control cell attachment, migration, proliferation and differentiation [11, 12]. Furthermore, this future generation of scaffolds should not behave as a permanent prosthesis but instead should perform as bio-resorbable temporary implants, allowing for the body to heal itself at the same rate as the implant degradation [11, 13].

In recent years a small number of synthetic biodegradable polymers, mainly polyesters containing glycolic (PLG) or lactic (PLL) acids and caprolactone (PCL) were approved by the Food and Drug Administration (FDA) for use in sutures [13]. Epicel™ (autologous keratinocyte skin graft to treat severe burn victims from Genzyme Biosurgery, Cambridge, MA), Carticel® (autologous chondrocyte transplantation to treat cartilage injury from Genzyme Biosurgery, Cambridge, MA) [14], MACI™ for matrix-induced autologous chondrocyte implantation (Genzyme Biosurgery, Cambridge, MA) where chondrocytes are supplied seeded onto a type I/III collagen scaffold secured to the skin injury with fibrin glue [15], and Apligraf (bovine collagen I matrix seeded with keratinocytes for wound care from Organogenesis, Canton, MA) [16], are products for cell therapy also available in the market. Other examples of products already commercially available are Atrigel® (Atrix Laboratories, Fort Collins, Co, USA) a system of biodegradable polymers for drug delivery [17] and the calcium phosphate based products Collagraft (Zimmer, Warsaw, IN; and Collagen Corporation, Palo Alto, CA) and ProOsteon (Interpore international, Irvine, CA) for bone applications [2]. However, since giving a detailed description of these and other products available in the market is not the purpose of this review we advise the reader to address other reports for more information [2, 13–17].

Despite the enormous research effort during the last few decades materials scientist have not fully developed a new generation of biocompatible biomaterials [13]. This limitation of tissue engineering to move forward from the laboratory into the clinic is the result of many issues, including legal, the need to develop functional blood vessel networks to nourish the new tissues mainly inside scaffolds, inability of the biomaterials to promote the formation of functional tissues, and many related issues [13]. For these reasons it is critical to develop the next generation of biomaterials that will address the limitations above. New approaches in the fields of bionanotechnology, protein engineering and bionano-fabrication will play a role in the development of these next generation biomaterials [18–20].

In this review we address some biopolymers already being used or with potential applications in regenerative medicine and tissue engineering, giving special focus to proteins and protein-based biomaterials. Additionally, we will also focus on the different approaches used for functionalization of these biomaterials in order to improve performance, mechanical efficiency, biocompatibility and degradability, usually with a goal towards control of these processes. This overview will be followed by a description of the novel design approaches, namely genetic engineering, enabling the synthesis of new protein-based biopolymers inspired in nature but without many of the drawbacks of their native counterparts when extracted directly from natural sources. Additional information can be found in recent reviews addressing the use of biomimetic materials in tissue engineering [21], the application of protein templates for tissue engineering [12], the synthetic modification of proteins and peptides [22] and the use of bioengineering for biomaterials design [19, 20].

2. Natural proteins for biomedical applications

The similarity between natural polymers and the macromolecules forming extracellular matrices suggests an innate ability for some of these polymers to interact with the cells and the biomolecules present in host tissues, inducing mild immunological reactions when compared with synthetic materials [11, 23, 24]. Natural polymers such as fibrin, fibronectin, collagen, elastin, silk, keratin, chitosan, alginate, amylose/amylopectin and hyaluronic acid are widely used in tissue engineering [23, 25]. Within the myriad of biopolymers present in nature, proteins are considered to be one of the most sophisticated groups in terms of chemistry [26]. Therefore some proteins with potential use in the biomedical field will be addressed in the next paragraphs. Table 1 addresses some of the basic features of the animal proteins described in this section.

Collagen is synthesized by fibroblasts and other cell types such as chondrocytes [27] and osteoblasts [28] and is the most abundant protein in the mammalian body, accounting for 20–30% of the total protein [29]. Its primary functions in tissues are to provide mechanical support [30] and to control cell adhesion, cell migration and tissue repair [31]. Collagens form a large family of triple helical molecules with about 28 different types described [32]. All collagens share the same triple-helical structure where three parallel polypeptides, α-chains, coil around each other forming a right handed triple helix chain. In animals these collagen triple helices are known as tropocollagen and its hierarchical organization into more complex structures generates the fibers and networks in tissues such as bone, skin tendons, basement membranes and cartilage [33, 34]. Collagen is easy to modify and process and its abundance, nonantigenicity, biodegradability, biocompatibility and plasticity make collagen a promising biopolymer for applications in the medical and pharmaceutical fields and tissue engineering purposes [30]. Reconstituted gels of Type I collagen are widely used for biomedical applications and its main sources are animal tissues such as skin and tendons [25, 35]. Collagen scaffolds have been extensively used for soft tissue repair [36], vascular [37] and dermal tissue engineering [38, 39], bone repair [40] and as a carrier for the

delivery of drugs [41] and biologically active molecules [42]. Additionally, collagens can also be used to fabricate microspheres for cell encapsulation [43] and drug loading for controlled release [44].

However, despite the wide range of applications collagens matrices lack the mechanical properties required for hard tissue during initial implantation. For this reason collagen is often blended with other materials, either synthetic [45] or natural [46], to overcome mechanical limitations [12].

Fibronectin is also a component of the extracellular matrix with important functions such as structural support and signalling for cell survival, migration, contractility, differentiation and growth factor signalling [47]. Fibronectin is synthesized by different cell types, such as fibroblasts and is secreted as a dimer with disulfide bonds formed between the 230–270KDa subunits. These subunits are formed by three types of repeating modules named type I, II and III [48]. Fibronectin is a multi-domain glycoprotein with a remarkable number of biological functions, many of which are mediated through interactions with integrins, such as via the RGD sequences present in fibronectin. Besides binding to cell integrins, fibronectin binds to other biologically important molecules such as heparin, collagen/gelatin and fibrin [49]. Since fibronectin is biocompatible and easily recognized by cell integrins, the use of fibronectin or domains of the protein to functionalize scaffolds for tissue engineering is often considered [50]. Polymeric scaffolds of chitosan [51, 52], collagen [53] and hyaluronic acid [54] have been modified with fibronectin to improve cell adhesion and proliferation.

Additionally, fibronectin-mimetic peptide-amphiphiles were used in the fabrication of nanofibers and gels with excellent cell adhesion properties [50]. Another strategy was to prepare fibronectin-terminated multilayer films of poly-lysine and dextran sulphate for the study of the spreading behaviour of human umbilical vein endothelial cells. The cells spread to a greater extent and in a more symmetric manner on the films coated with fibronectin, suggesting that such fibronectin coated films may represent a promising strategy to control cell interactions with the materials in tissue engineering [55].

Together with collagen and fibronectin, **elastin** is also part of the core architecture supporting cell adhesion and growth [56]. Elastin fibers are mainly present in connective and vascular tissues, the lungs and skin. Elastin is a polymer of tropoelastin monomeric precursor and elastin fibers are an important component of the extracellular matrix to impart elasticity to organs and tissues. Hydrophobic domains present in the elastin sequence are responsible for these elastic properties [57, 58]. Elastin also has chemotactic activity, inducing cell proliferation and regulating cell differentiation, with the specific binding of integrin $\alpha_v\beta_3$ to the C-terminus in tropoelastin [59]. Due to its characteristics elastin is of interest for drug delivery and tissue engineering and has been used in the fabrication of hybrid materials in combinations with collagen [60], polycaprolactone (PCL) [61] and silk [62] for the production of vascular grafts [63], hydrogels [64], bone repair [65] and for drug delivery [66]. However, the crosslinking that occurs between the water-soluble tropoelastin monomers to form the insoluble and stable elastin fibers limits the use of elastin from animal origin [56]. Therefore artificial proteins incorporating elastin-like peptides have been of interest for the development of new protein-based biomaterials [67, 68] with properties similar to native elastin [69].

Fibrin is another example of a specialized extracellular matrix protein with potential application for tissue engineering. However, unlike collagen, elastin and fibronectin, fibrin networks form mostly during blood clotting. Fibrin is the result of fibrinogen polymerization in the presence of thrombin [70]. Fibrinogen is a 340 kDa protein present in plasma formed

by pairs of three different polypeptides, Aα, Bβ and γ, held together by disulfide bridges [71]. Fibrin and fibrinogen are two important components in blood clotting, fibrinolysis, cellular and matrix interactions, inflammation, wound healing and neoplasia [72]. In the particular case of clot formation, thrombin cleavage both Aα and Bβ chains at their N-termini, leading to the exposure of polymerization sites in both chains [73]. Subsequently the combination of these polymerization sites leads to the formation of double-strand twisted fibrils. These fibrin protofibrils undergo lateral aggregation and form branches, producing a three dimensional network [74]. Blood clots are further stabilized by covalent bonds formed by the plasma transglutaminase, factor XIII, making the clot more mechanically stable and less susceptible to enzymatic digestion [75]. Fibrin is a viscoelastic polymer and is used clinically as a medical adhesive; fibrin sealants are FDA approved.

Furthermore, fibrin is also used for skin repair, replacing sutures and staples in fixation of skin grafts promoting a better wound healing [76], and in the transplantation of keratinocytes in burned patients [35]. Fibrin is also a promising biopolymer for applications in tissue engineering, in the repair of damaged tissues [77, 78], and drug delivery, as a carrier for growth factors [79]. Additionally, two proteinaceous components of the extracellular matrix, laminins and vitronectin, are mainly used to coat synthetic and natural polymer-based materials to improve cellular response. **Laminins** are cell adhesion glycoproteins localized in the extracellular matrix of the basement membrane and are able to bind to other matrix proteins [80]. Recently, lamimin-derived peptides have been used as coatings to induce the adhesion of different cell types such as hepatocytes [81] and human dermal fibroblasts [82]. Also, these peptides are being studied for drug delivery in the development of targeting drug-loaded systems for cancer treatments [83]. **Vitronectin** is a multifunctional glycoprotein present in the extracellular matrix where it binds to glycosaminoglycans, collagen, plasminogen and urokinase-receptor and its RGD allows it to mediate the adhesion and spreading of cells [84]. This multipartner binding makes vitronectin an attractive biopolymer for tissue engineering and to induce cell attachment when used as a surface coating [85, 86].

The proteins described above are extracellular matrix proteins and have been more commonly used for tissue engineering and regenerative medicine applications. However, in the past few years other proteins have also emerged as potential biopolymers for the fabrication of new biomaterials, such as **Keratin** [87]. Moreover, since it is a protein shared by all mammals with a highly conserved amino acid sequence it is expected to offer good cell and tissue responses [88]. keratin fibers are hierarchically structured proteins present in hard and filamentous structures, such as hairs, horns and nails [87]. The presence of a LDV cell binding domain in keratin amino acid sequence [87] suggests utility for the fabrication of scaffolds for tissue engineering. Keratin based biomaterials have been used to support adhesion, spread and growth of L929 fibroblast cells [89], and the growth and differentiation of osteoblasts (MC3T3-E1) [90]. Keratin films have an inhibitory effect on the IgE receptor-stimulated histamine release from mast cells, making it suitable for use in antiallergenic materials [91].

As collagen and keratin, **silk** is another example of a hierarchically structured fibrous protein. Silk is characterized by its outstanding mechanical properties out-competing high performance man made fibers such as Kevlar, nylon and high-tensile steel, and by its self-assembly leading to fibers with a complex hierarchical arrangement [26]. Silk-protein-based fibers are produced by insects [92] and spiders [93] which use it for different ends such as cocoon and nest construction. However, despite the multitude of functions and different protein structures, many silk-based fibers have similar amino acid compositions and high levels of crystallinity. Silkworm silk produced by the silkworm species *Bombyx mori* is the most well studied silk protein [92]. The silk fiber is formed by two microfilaments

embedded in glue-like glycoproteins named sericin which works as a coating. Each microfilament results from the assembly of a hydrophobic ~370 kDa heavy-chain fibroin protein, a relatively hydrophilic ~25 kDa light-chain fibroin and a 30 kDa P25 protein [94]. Spider dragline silk has a slightly different structure with a core filament formed by two spidroin molecules, major ampullate spidroin protein 1 (MaSp1) and 2 (MaSp2), coated by glycoproteins and lipids [95]. The remarkable mechanical features of the different types of silk are in part due to the presence of α-helix and β-turns, responsible for its elastic properties. These elastic domains alternate with β-sheet motifs which confer toughness to silk fibers. The strong molecular cohesion occurring with amide-amide interactions in the β-sheet crystalline regions is thought to be responsible for the remarkable stiffness of silk fibers [96]. In *B. mori* silk, the hexapeptide repeat GAGAGS is involved in the formation of the β-sheets. In spider silk besides GA sequences there are also poly-Ala blocks and both motifs contribute for the formation of anti-parallel β-sheets [96]. These poly-A and GA motifs are embedded in amorphous regions formed by either GGX (X can be Tyr, Leu or Gln) or GPGXX motifs believed to be responsible for the elastic features [97]. The outstanding mechanic features and biocompatibility are reasons why silk has been used through the millennia in such diverse applications as hunting, fabrication of paper, wound dressing, textiles and sutures [98]. With new technologies in the fields of polymer synthesis and processing, silk continues to be an important topic of research for biomaterial and biomedical research. In the case of *B. mori* silk, sericulture provides the product used by the textile industry and in medical sutures [93]. Additionally, this silk is being studied for tissue engineering in the form of scaffolds for a range of tissue needs, such as corneal regeneration [99, 100], cartilage repair [101, 102], vascular grafts [103, 104], bone regeneration [105, 106] and drug delivery [107, 108]. As mentioned above *B. mori* silk is available in large supplies from sericulture, and is therefore most commonly used for the above studies. In the case of spiders, it is difficult to breed spider species due to their cannibalistic behaviour. With the advance of biotechnology tools it is now possible to bioengineer spider silk genes to produce spider silk-like proteins [109], such as for tissue engineering [110], cell culture [111], nerve regeneration [112, 113] and wound dressings [114].

Mussel adhesive proteins (MAPs) are produced by marine mussels and used in the formation of the byssal threads which allow the animal to anchor to substrates. A common feature to all the adhesives produced by mussels is the presence of the amino acid 3,4-dihydroxyphenyl-L-alanine (DOPA). DOPA residues are key elements for the chemisorption to substrates underwater and the crosslinking process within the adhesive molecules [115]. These natural adhesives display outstanding properties in terms of function under harsh marine environments with wide temperature, salinity and humidity fluctuations and the mechanical effects of tides, waves and currents [116]. These remarkable properties make MAPs attractive biomaterials as bioadhesives. MAPs have been used as bioadhesives for cells [117] and as self-adhesive micro-encapsulated drug carriers for biotechnological, tissue engineering and biomedical applications [118]. MAP derivatives were also used in the fabrication of adhesive-coated meshes as wound sealants, replacing tradition sutures, staples and tacks [119].

The proteins addressed above are widely used for tissue engineering and biomedical applications and can be obtained from animal sources. Moreover, the majority of proteins used in the development of new scaffolds for tissue engineering are extracted from natural sources. In this way, in most cases these polymers need further modifications to make them more suitable for different biomedical applications. The next section refers to physical and chemical approaches used for the functionalization of these biomaterials.

3. Techniques for the functionalization of protein-based biomaterials

The properties of protein-based biomaterials can be improved by combining them with bioactive molecules to enhance *in vitro* and/or *in vivo* functions. The surface of protein-derived scaffolds can be modified by physical adsorption, physical entrapment (encapsulation) or by chemical modification. These techniques are commonly used to functionalize protein-based biomaterials with different biologically active molecules, such as growth factors and antibiotics, improving cell and tissue responses.

Physical adsorption is a simple immobilization procedure and is frequently used to attach bioactive molecules such as extracellular matrix proteins or growth factors to the surface of scaffolds by dip coating [120]. Adsorption efficiency is dependent on the physical and chemical properties of the material, including wettability, surface topography, functional groups, pH and electrical charge, among other factors [121]. Many biomaterials are hydrophobic, therefore, methods are needed to enhance wettability to make them more hydrophilic. Physical methods such as bombardment with ions, UV light and plasma modification are used to disrupt chemical bonds between carbon and non-carbon atoms generating unsaturated bonds and radicals which react with oxygen, increasing hydrophilicity and enhancing reactivity towards biological molecules [121]. Natural polymers have the advantage of being rich in reactive chemical groups (hydroxyl, carboxyl, amide) which make them more hydrophilic and capable of interacting with bioactive molecules. Collagen and silk are examples of protein-based materials that have been functionalized through adsorption of bioactive molecules, including bone morphogenetic proteins (BMPs) [122, 123], basic fibroblast growth factor (bFGF) [124], vascular endothelial growth factor (VEGF) [125] and therapeutic compounds such as antibiotics [126] and heparin [127] as it is summarized in Table 2. In most of these studies the protein-based scaffolds were soaked in a solution containing the bioactive component. In other cases the proteins were blended with the bioactive molecule in solution and then cast to form scaffolds [128].

Since adsorption is based on relatively weak or moderate electrostatic, van der Waals, hydrogen and hydrophobic interactions the binding stability of the adsorbed molecules can vary depending on environmental conditions. In this way, changes in pH, ionic strength and adsorbed species concentration of the surrounding medium can result in an uncontrolled release of the immobilized species [120]. For example, bone morphogenetic proteins (BMPs) tend to diffuse away from the fracture area and high doses are required to induce the desired osteogenic response. The release profile of BMP-2 from collagen sponges shows an initial burst during the first 10 minutes, where the carrier loses around 30% of the BMP-2, followed by slow release during the next 3 to 5 days. This initial burst release can cause clinical complications, such as ectopic bone formation, soft tissue hematomas and bone resorption [129, 130].

To overcome these issues, **covalent immobilization** has been widely used since it has the advantage of providing stable attachment of bioactive agents to polymeric scaffolds. With proper design, covalent conjugation has proven to be a very effective strategy to control the release profile of the immobilized agent since these molecules are retained for longer time periods at the delivery site, when compared with adsorption [11]. Carbodiimide coupling is broadly used in protein chemistry to react activated surface carboxylic acid groups from protein-based scaffolds with the amines present on the peptide or protein to be immobilized [131, 132]. Carboxylic groups are activated by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) mixed with either N-hydroxysuccinimide (NHS), dicyclohexyl-carboiimide (DCC) or carbonyl diimidazole (CDI) [131, 132]. This basic protein chemistry has been extensively used to immobilize molecules as it is shown in Table 2, including

BMPs and RGD peptides onto silk and collagen scaffolds. A drawback of this coupling method is the difficulty in characterizing the new peptide-protein scaffolds, due to the background noise from the protein scaffold itself, making it difficult to measure the signal coming from the small amount of peptide immobilized on the scaffold surface in order to quantify how much peptide was immobilized [132]. Another drawback can be the presence of reactive amine groups aside from the N-terminal amine. These reactive side groups need to be protected, followed by deprotection after the coupling chemistry is carried out, although the use of harsh conditions can affect the biological activity of the immobilized molecules [131].

Glutaraldehyde, polyethylene glycol diacrylate and hexamethylene diisocyanate can be used to bridge the amine groups present in the peptide or protein to be immobilized and in the protein based scaffolds [132, 133]. Glutaraldehyde has been used to couple insulin [134] and lipase [135] onto silk scaffolds and to crosslink blends of collagen and silk [136]. However, the potential release of toxic residual molecules formed during the crosslinking process is a concern if these biomaterials are to be used for biomedical applications [133].

Encapsulation of bioactive molecules within protein matrices has also been explored as a method to control the release of bioactive agents. In many cases chemical modifications are required in order to have better control over the release profile of the encapsulated molecules. Crosslinked gelatin microspheres later impregnated with basic fibroblast growth fact (bFGF) and loaded into collagen sponges were used in order to have controlled release of bFGF at a defect site [137] (Table 2). Furthermore, crosslinked collagen microspheres loaded with bovine serum albumin (BSA) and nerve growth factor were prepared and release profiles assessed [44]. In both studies collagen microspheres had to be crosslinked in order to reduce the initial burst and attain better control of protein release. EDC and NHS were also used as coupling reagents to covalently bind 2,3-dihydroxybenzoic to gelatin microspheres, which were incorporated into a reconstituted collagen scaffold for a wound dressing [138]. Silk microspheres were used for the encapsulation of bioactive proteins and other molecules, exploiting the self-assembly properties of silk to control the release profile [139].

Many formulations and delivery strategies have been explored in order to achieve functionalization and sustained release of different molecules. However, in the particular case of bioactive proteins loaded into protein-based scaffolds, protein structure and topology must be considered in order to prevent protein denaturation, as a consequence of the adsorption or immobilization processes, and protein aggregation during the release period which can result in the loss of bioactivity [11, 140]. Proteins in denatured forms are often antigenic and can induce immunogenic reactions with negative clinical consequences [140].

Most of the methods being used for functionalization of polymeric structures and drug release have some disadvantages and new strategies are clearly needed. Advances in the fields of self-assembly and biotechnology, mainly via recombinant DNA approaches, can offer some important options to address the deficiencies noted above, to help in the development of the next generation of biomaterials. The importance of recombinant DNA technology for the development of new protein based biomaterials will be the focus of the next section.

4. Recombinant proteins for tissue engineering

Since mammalian tissues are the main source of materials such as collagen, gelatin, fibrin and elastin there are concerns with disease transmission and immunogenic responses in *in vivo* studies, as well as batch-to-batch variability [19, 141]. To overcome these limitations, peptide synthesis and recombinant DNA protein methodologies have been explored.

Chemical synthesis can be a quick and efficient method to fabricate short peptides in relatively small quantities [142]. However, the synthesis of peptide sequences with more than 35–40 amino acids is not feasible due to a drop in yield and efficiency paralleled by an exponential increase in cost [143]. Recombinant DNA technology provides well established protocols for cloning, mutation and gene fusion in different host cells for the expression of peptides and proteins with a broad range of sizes [144]. Furthermore, the increased efficiency in making synthetic oligonucleotides and the use of standardized kits and protocols for cloning and protein expression make the transgenic production approach more cost-effective for large scale protein production [144]. Besides engineering biological components already present in nature as shown in Table 3, the field of synthetic biology is also focused on the design of new peptides and protein sequences. This can be achieved by establishing new artificial self-assembling peptides or by fusing together different bioactive domains or protein motifs that are not otherwise found together in nature. Table 4 gives an overview of the studies published during the past few years using this approach [144]. Since genetic engineering offers the possibility of altering the amino acid sequence of the expressed protein by adding or substituting codons, it is possible to generate alternative sequences with tunable properties that can be used as promising biomaterials for medical applications.

Below we will address some of the proteins that have been effectively cloned and expressed in different recombinant systems. The potential of genetic engineering to be used as a tool for the functionalization of biopolymers with different bioactive peptides through the synthesis of new fusion proteins will also be discussed.

Collagen has been cloned and expressed in recombinant systems (Table 3). The use of recombinant collagen has benefits since it can be a safe product with useful self-assembly features [144] and the possibility of being functionalized with bio-instructive domains [19] such as cell adhesion ligands [141]. Over the past 20 years recombinant systems for the large scale-production of recombinant collagen have been developed and optimized. Recombinant collagen has been expressed in mammalian cells, insect cells, *Escherichia coli* (*E. coli*), transgenic tobacco, mice and silkworm [145]. From these recombinant hosts only the mammalian cells expressed collagen with 4-hydroxyproline content identical to native collagen. However, since the level of protein production was low (0.6–20 mg/L) this system was not commercially viable [146]. Since the production cost in yeast and *E. coli* is much lower than in mammalian cell culture, a multigene expression technology was adopted in order to overcome the absence of the enzyme prolyl 4-hydroxylase, an essential element in the synthesis of fully hydroxylated collagens [146]. The absence of this enzyme leads to non-triple-helical and non-functional collagen molecules, which are unstable below physiological temperatures and thus unsuitable for medical applications. Hence, the multigene expression approach based on the co-expression of procollagen polypeptide chains and α- and β-subunits of proyl 4-hydroxylase using the yeast, *Pichia pastoris*, was developed [147]. Collagen types I, II and III were expressed with a 4-hydroxyproline content identical to the native human proteins and expression levels of 0.2 to 0.6 g/L in 2 L bioreactors were achieved [147]. The use of recombinant collagen as a gel has been reported for chondrocytes [148], as a microcarrier [149], as corneal substitutes [150] and for bone regeneration applications [151]. Furthermore, customized collagen-like peptides formed with tandem repeats of the D4 domain of human collagen type II, a critical sequence for supporting the migration of chondrocytes, were also reported [140]. Chondrocytes seeded on polyglycolic acid scaffolds coated with this collagen-like protein formed cartilaginous constructs with superior properties to the scaffolds coated with native type II collagen [152]. These advances highlight the importance of recombinant DNA technology in the synthesis of proteins with applications that until now have only been available from animal sources.

Recombinant DNA technology was particularly advantageous in the expression of large and repetitive proteins such as silk. As in the case of collagen, different expression hosts have been explored for the biosynthesis of spider silk (Table 3). Major ampullate silk was successfully expressed by bovine mammary epithelial cells, hamster kidney cells, insect cells and in the milk of transgenic goats, generally with low yields [153]. However, bacteria can be grown at large scales and have the advantage of being easier to handle and more cost-effective. Therefore, *E. coli* has been actively pursued as an expression host for spider silks. Since bacterial hosts have distinct codon usages, silk sequences from different spider species were reverse transcribed into cDNA, using the *E. coli* codon preferences, and double stranded oligonucleotides coding for different domains of silk proteins were prepared [154]. These double strand oligonucleotides were then assembled into synthetic genes coding for silk proteins [153]. This cloning strategy was employed with successes for the expression of *Nephila clavipes* consensus sequence for major ampullate silk protein 1 (MaSp1) and MaSp2 [155] and the flagelliform silk protein [156] from the same species. Cloning and expression in *E. coli*, of both major ampullate silks ADF-3 and ADF-4 from the species *Araneus diadematus* was also reported (Table 3) with yields between 140 and 360 mg/L [157]. Besides *E. coli*, other hosts for the cloning and expression of spider silks have also been explored. The yeast *Pichia pastoris* is considered an attractive host for the expression of recombinant proteins since this expression system is well developed for industrial fermentation, reaching high cell densities using low-cost media. For these reasons it was successfully used for the expression of spider silk dragline using genes of up to 3,000 codons with no evidence of truncated synthesis, a common occurrence in *E. coli* host [158]. Plants such as tobacco and *Arabidopsis thaliana* are also being explored as transgenic host systems for silk proteins, with yields of 2% in tobacco leaves, 8.5% in *A. thaliana* leaf apoplasts and 18% in the endoplasmic reticulum of seeds [153]. Similar approaches as above for collagens and silks have been applied to the fabrication of recombinant elastin-like proteins that mimic native elastin (Table 3) [56]. These new protein polymers have a modular structure formed with repeats of the pentapeptide (VP-Xaa-Yaa-G)n where Xaa is either G or A and Yaa can be any residue but P. These recombinant elastin-like proteins are capable of reversible temperature-dependant self assembly in aqueous medium [67]. This feature allows for the purification of protein based upon temperature-induced aggregation. Elastomeric pentapeptides with up to 251 GVGVP repeats were soluble in low ionic solution at temperatures below 25ºC [159]. Above this temperature the polymer hydrophobically folds into β-spiral structures that further aggregate due to hydrophobic associations. These aggregates can then be collected by selective centrifugation. This methodology allows for facile purification [160, 161]. Moreover, elastin-like polypeptides (ELPs) can be used as a purification tag. The fusion of ELPs with other proteins exploits the inverse temperature transition of ELPs and provides a simple method for the isolation of a recombinant ELP fusion proteins by cycling the protein solution through the soluble and insoluble phases using inverse transition cycling [162–164]. ELP tags can be cleaved by a pH shift and removed by a final thermal precipitation [164].

Additionally there is the possibility of amino acid substitutions in the pentapeptide repeats [165] and previous studies have shown that the replacement of G in $(VPGVG)_n$ by A in $(VPAVG)_n$ leads to mechanical changes in the protein from elastic to plastic [67, 166]. The physical crosslinking resulting from this amino acid replacement leads to a more plastic matrix with a Young's modulus two orders of magnitude higher than in the case of $(VPGVG)_n$ [166]. Also, physical crosslinking has advantages over chemical crosslinking since it allows for easy processing, avoids the use of chemical reagents and excludes the need of removing unreacted intermediates [167]. Synthetic amphiphilic block copolymers with distinct block polarity composed of hydrophilic and hydrophobic segments can also be generated [67]. These block copolymers exhibit tunable mechanical and amphiphilic properties dependent on the amino acid substitution. The flexibility of these block

copolymer designs extends the range of applications from micelles formed by self-assembly of amphiphilic sequences for drug delivery, to temperature responsive hydrogels for cell encapsulation and coatings of medical devices to improve host responses [168–170]. Genetic engineering also offers the possibility of enriching the sequences of proteins to improve their biological activity by fusing them with other protein motifs with specific bioactivities (Table 4). Initial elastin matrices for cell adhesion showed that cells did not adhere to these biomaterials [171]. RGD and REDV cell adhesion peptide sequences were inserted into the elastins leading to a dramatic increase in cell attachment [169, 170]. Silk-based block copolymers were also engineered to carry an RGD cell binding domain for intracellular gene delivery. The presence of labelled DNA inside cells was detected by confocal laser scanning microscopy and demonstrates the potential of these silk bioengineered block copolymers as highly tailored gene delivery systems [172]. The addition of a recognition site for an enzyme with proteolytic activity can also be incorporated into the sequences, favouring biomaterials degradation [173]. The fusion of the *N. clavipes* consensus sequence for MaSp1 with proteins such as dentin matrix protein and bone sialoprotein, involved in calcium phosphate deposition in teeth and bone [174, 175], respectively, also had positive results from a biomaterials perspective [176, 177]. In both fusion proteins the silk domain retained its self assembly properties and the dentin matrix protein and bone sialoprotein domains maintained their ability to induce the deposition of calcium phosphates. These results demonstrated the potential of chimeric proteins for applications in tissue engineering and regenerative medicine for the design of new protein-based scaffolds for bone regeneration [176, 177].

Furthermore, promising results were also obtained when the *N. clavipes* consensus sequence for MaSp1 was fused with antimicrobial peptides, namely neutrophil defensins 2 and 4 and hepcidin, using a step-by-step cloning methodology [178]. The cloning and expression of these new fusion proteins expanded these chimera or fusion approaches to include antimicrobial-functionalized protein-based biomaterials [178] offering a path forward in reducing the use of antibiotics to prevent infection in implants and in the design of a new generation of protein-based materials bioengineered to prevent the onset of infections.

Other proteins have also been expressed as fusion proteins with biological activity such as FGF2-FNIII9-10 formed by a fibronectin fragment FNIII9-10 connected to the carboxy terminus of fibroblast growth factor 2 (FGF-2) [179]. Previous studies reported the synergistic effect of fibronectin and FGF-2 on osteoblast adhesion. The FGF2-FNIII9-10 fusion protein showed a significant increase in cell adhesion and proliferation when compared with FNIII9-10 alone [179]. The cell-binding domain of human fibronectin was also fused with epidermal growth factor (EGF), important in tissue regeneration to accelerate wound healing and enhance cell proliferation. The new construct, designated as C-EGF, had both cell-adhesive and EGF activity and the recombinant construct may be an effective drug delivery system for EGF in therapeutic situations [180]. EGF polypeptide was fused with collagen type III and the new construct retained the triple helix of collagen and the mitogenic activity of EGF, suggesting that this protein could be used as a biocompatible, biodegradable and adhesive fibrous mitogen for tissue regeneration [181].

The examples outlined above highlight the potential of synthetic biology in the synthesis of biopolymers for tissue engineering and regenerative medicine (Figure 1).

5. Conclusions

Genetic engineering makes it possible to develop new biopolymers with a complexity and functionality resembling natural polymers formed in nature. By using synthetic DNA it is possible to combine different functional domains for a fusion protein, merging cell adhesion

and migration, mechanical properties and antimicrobial factors, towards multifunctional biomaterial systems. This approach eliminates the need to use chemical methodologies for covalent binding of bioactive motifs or crosslinking, which can have drawbacks of protein denaturation and residuals with toxicity. Although there has been a significant progress in exploiting genetic engineering for tissue engineering and regenerative medicine purposes during recent years, there remains a lot to be explored in order to take full advantage of the outstanding potential of genetic engineering to be used as a tool in the development of the next generation of custom-design biomaterials.

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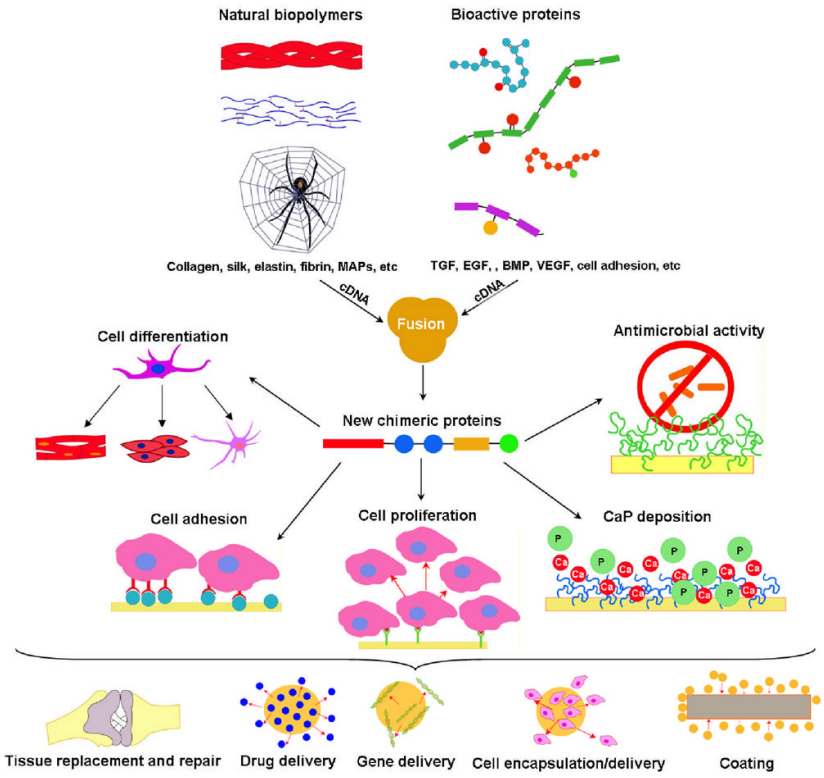

Fig. 1.

Scheme highlighting some of the features and applications of chimeric protein-based biomaterials synthesized through recombinant DNA technology.

Table 1

Basic features of some proteins with potential applications in the biomedical field.

Protein	Main functions	Basic structure	Relevant properties
Collagen [31, 34]	Structural protein in tissues such as connective tissue, tendon, skin, bone and cartilage	Three parallel polypeptide chains formed by GXY (G - glycine, X - usually proline, Y - usually 4-hydroxyproline) repeats and arranged in triple helix	Biodegradability, low antigenecity and biocompatibility
Fibronectin [47, 49]	Structural support and cell signalling	Dimer of two non-identical polypetide chains bonded at the carboxyl end by disulfide bonds	Multi-domain protein with cell (RGD motif), collagen and fibrin binding motifs
Elastin [56, 182, 183]	Structural protein found predominantly in connective tissue of arteries, ligaments, skin and lung	Cross-linked units of tropoelastin formed by hydrophobic (often 3 to 6 repeats of GVGVP, GGVP and GVGVAP) and hydrophilic lysine domains	Temperature dependent self-assembly and phase separation behaviour
Fibrin [72, 184]	Blood clotting, fibrinolysis, cellular and matrix interactions, inflammation and wound healing	Resultant from the polymerization and crosslinking of fibrinogen units after thrombin cleavage	Growth factor binding and interaction with cells such as platelets, leucocytes, fibroblasts and endothelial cells
Laminins [80]	Major components of basement membranes underlying epithelial and endothelial cells and embedding Schwann, muscle and fat cells	Heterotrimers of one β, one α and one γ chain, which represent different gene products	Self-assembly and binding to several matrix proteins and integrins
Vitronectin [84]	Regulates clot formation and immune response, provides biological cues for cell adhesion, migration and proliferation and extracellular anchoring	In human blood is found as a single chain or as a dimer while in the extracellular matrix exists as a disulfide-linked vitronectin multimer	Multi-domain protein with an RGD motif to mediate the attachment and spreading of cells and binding motifs for collagen, heparin, plasminogen, glycosaminoglycan and fibrin binding motifs
Keratin [88, 185]	Structural protein in the cytoskeletons of vertebrate epithelial cells and epidermis appendages such as hair, nails and wool	Formed by α-helical coiled-coil dimers assembled into 10 nm wide filaments	Biocompatibility, good cell attachment and growth
Silk [93, 98]	Building element of many arthropod nests, cocoons and prey traps	Highly repetitive core domain of alternating poly-A hydrophobic and G rich hydrophilic motifs	Self-assembly and remarkable mechanical properties
Mussel adhesive proteins (MAPs) [119]	Substrate adhesion	Repetitive sequence, with molecular weights ranging between 5 and 120 kDa and high presence of 3,4-dihydroxyphenyl-L-alanine (DOPA)	Function over a wide range of temperatures, humidity and salinity and form permanent bonds to a wide variety of surfaces

Table 2

Summary of protein based scaffolds functionalized with different bioactive molecules.

Protein matrices	Modification process	Modifying molecule	Application	References
Collagen	EDC/NHS covalent immobilization	VEGF	Vascularisation/angiogenesis	[186, 187]
		FGF/VEGF	Vascularisation/angiogenesis	[188]
		Heparin	BMP/FGF/PDGF delivery system	[189–191]
	Traut's Reagent and Sulfo-SMCC covalent immobilization	poly-Histidine antibody	BMP delivery system	[192]
		VEGF	Vascularisation/angiogenesis	[193]
	Adsorption	BMP-2	BMP delivery system	[194]
		FGF	Cartilage regeneration/Growth factor delivery	[195, 196]
	Microsphere encapsulation	BMP-7	BMP delivery system	[197]
		VEGF	Vascularisation/angiogenesis	[198]
Gelatin	EDC covalent immobilization	TGF-beta	Cartilage regeneration	[199]
	Adsorption	TGF-beta/IGF	Cartilage regeneration	[200]
		FGF	Growth factor delivery	[195]
		Fibronectin	Cartilage regeneration	[201]
	Microsphere encapsulation	TGF-beta	Chondrogenesis/cartilage regeneration	[202, 203]
		BMP-2/VEGF	Angiogenesis and osteogenesis	[204]
		BMP-2	Growth factor delivery	[205]
Fibrin	Microsphere encapsulation	FGF	Angiogenesis	[206]
		BMP-2	Bone regeneration	[207, 208]
	Patterning immobilization	FGF-2	Tissue engineering	[209]
Heparin	Michael type addition	BMP-2	Bone/ligament regeneration	[210]
		HGF	Hepatocyte differentiation	[211]
Silk	Cyanuric chloride immobilization	Lactose	Hepatocyte attachment	[212]
	Crosslinking	Gelatin	Tendon tissue engineering	[213]
	Adsorption	FGF	Growth factor delivery	[124]
		Gelatin	Drug/Growth factor delivery	[214]
		Collagen/chodroitin-6-sulfate/hyaluronan	Tendon tissue engineering	[215]
		Collagen	tendon tissue engineering	[216, 217]
		BMP-2	Bone regeneration	[218]
	Microsphere encapsulation	IGF	Drug/Growth factor delivery	[219]
	Blend	Gelatin	Tissue engineering	[220]
Silk/collagen	Adsorption	SDF-1	tendon tissue engineering	[221]

Table 3

Biopolymers expressed in recombinant systems and their potential uses.

Protein	Expression system	Advantages/Applications	References
Collagen I	Transgenic corn	Food and pharmaceutical industries	[222]
	Yeast *Pichia pastoris*	Identical 4-hydroxyproline content to human collagen; medical applications such as corneal replacement	[147, 150, 223]
	Yeast *Saccharomyces cerevisiae*	Study of collagen expression and maturation	[224, 225]
	Mammalian HT1080 cells	Optimization of recombinant collagen expression and isolation methodology	[226, 227]
	Insect cells	Optimization of recombinant collagen expression and isolation methodology; Structural studies	[228, 229]
	Mammalian, mouse milk	Optimization of recombinant collagen expression	[230, 231]
	E. coli JM109 strain	Large quantities production/Therapeutic, biomaterial, or bioengineering applications,	[232]
	E. col	Bone tissue engineering	[151]
Collagen II	Yeast *Pichia pastoris*	Identical 4-hydroxyproline content to human collagen	[147]
	Insect cells	Optimizing recombinant collagen expression systems	[233]
Collagen III	Yeast *Pichia pastoris*	Higher production level; Identical 4-hydroxyproline content to human collagen; Scientific and medical applications such as corneal replacement	[147, 150, 234]
	Yeast *Saccharomyces cerevisiae*	Optimizing recombinant collagen expression systems	[235]
	Insect cells	4-hydroxyproline content similar to human collagen; Study of collagen chain association and folding	[236, 237]
	Silkworm	Viable expression system for bulk protein expression	[238]
Collagen V	Mammalian cells	Structural studies	[239]
Collagen VI	Mammalian cells	Collagen and heparin binding studies	[240]
Collagen VII	Mammalian cells	Study of dystrophic epidermolysis genetic disorder	[241]
Collagen X	Mammalian HEK293 cells	Optimizing recombinant collagen expression systems	[242]
Collagen XI	*E. coli* BL21	Study the regulation of collagen fibrillogenesis	[243]
Collagen-like protein	Mammalian HT1080 cells	Biomedical applications	[152, 244]
Gelatin-like proteins	Yeast *Pichia pastoris*	Biomedical applications	[245]
Elastin-like peptides	Yeast *Pichia pastoris*	Optimizing cloning and expression process	[246]
	E. coli strain BL21-Gold	Vascular replacement; Tissue engineering, controlled drug release and cell encapsulation; Biomedical applications	[167, 247–253]
	E. coli BLR strain	Biomedical applications	[254]
Spider silk major ampullate from *Nephila clavipes*	*E. coli* RY-3041	Structural studies/Biomedical applications	[255, 256]
	E. coli SG 13009pREP4	Structural studies/Biomedical applications	[155]
	E. coli BL21	Structural studies/Biomedical applications	[257–259]
	E. coli M109 strain	Structural studies/Biomedical applications	[260]
	Yeast *Pichia pastoris*	Structural studies/Biomedical applications	[158]
Spider silk major dragline proteins ADF-3 and ADF-4 from *Araneus diadematus*	*E. coli* BLR strain	Structural studies/Biomedical applications	[157]

Protein	Expression system	Advantages/Applications	References
Spider silk flagelliform from *Nephila clavipes*	*E. coli* BL21 strain	Structural studies/Biomedical applications	[156]
Spider silk like proteins - NcDS, (SpI)7 and [(SpI)4/(SpII)1]4	*E. coli* BL21 strain	Structural studies/Biomedical applications	[261]
Fibrinogen	Mammalian cells	Fibrin sealant	[262]
	Yeast *Pichia pastoris*	Fibrin sealant	[263]
Fibronectin	*E. coli*	Cell adhesion	[264, 265]

Table 4

New chimeric proteins with potential application in the biomedical field.

Fusion protein	Expression system	Applications	References
R136K (FGF-1 mutant) + collagen biding domain	*E. coli* BL21 (pLysS) strain	Selective binding to collagen and potent angiogenic, mitogenic and chemotactic activity for endothelial cells	[266, 267]
VEGF + collagen biding domain	*E. coli* BL21 strain	Improve diabetic wound healing	[268]
FGF + fibronectin cell binding domain	*E. coli* JM109 strain	Stimulates angiogenesis, biomedical applications/tissue engineering	[269]
FGF + collagen binding domain	*E. coli* BL21 strain	Delivery systems/Biomedical applications/ Tissue engineering	[270]
FGF + glutathione S-transferase (GST-bFGF)	*E. coli*	Stimulate the growth of human umbilical vein endothelial cells	[271]
FGF2 + Fibronectin (FGF2-FNIII9-10)	*E. coli* TOP10 strain	Delivery of bioactive molecules	[179]
EGF + collagen binding domain	*E. coli* BL21(DE3) strain	Delivery systems/Biomedical applications/ Tissue engineering	[270]
EGF-collagen	Insect cells	Tissue engineering applications	[181]
EGF + immunoglobulin G (IgG) Fc region (EGF-Fc)	*E. coli* BL21 strain	Cell adhesion	[272]
Silk + elastin (SELP-47 K)	*E. coli*	Promote cell attachment and growth/Tissue Engineering	[273]
Spider silk + dentin matrix protein	*E. coli* RY-3041 strain	Biomedical applications/Tissue engineering	[177]
Spider silk + bone sialoprotein	*E. coli* RY-3041 strain	Biomedical applications/Tissue engineering	[176]
Spider silk + antimicrobial domain (HNP-2, HNP-4 and hepcidin)	*E. coli* RY-3041 strain	Biomedical applications/Tissue engineering	[178]
Bombyx mori silk + RGD + elastin (FES8)	*E. coli* BL21 strain	Biomedical applications	[274]
RGDS + silk fibroin (RGDSx2 fibroin)	Silkworm	Facilitate chondrogenesis	[275]
Collagen + GYIPEAPRDGQAYVRKDGEWVLLSTFL	*E. coli* BL21 strain	Stabilize the triple helix formed in the proteins/Biomedical applications	[276]
BMP-2 + collagen-biding domain	*E. coli* BL21 strain	Bone repair	[277–279]
TGF-B1-F1 and TGF-B1-F2 + collagen binding domain	*E. coli*	Biomedical applications/Tissue engineering	[280]
hbFGF-F1 and hbFGF-F2 + collagen binding domain	*E. coli*	Biomedical applications/Tissue engineering	[281]
PDGF + collagen binding domain	*E. coli* BL21 strain	Tissue regeneration and wound repair	[282]
Fibronectin III7–10 + cadherin 11 EC 1–2	*E. coli* Rosetta-gami strain	Orthopaedic regeneration	[283]
Fibronectin cell binding domain-EGF (C-EGF)	*E. coli* HBIOI strain	Drug delivery	[180]
Fibronectin cell binding domain-EGF (FNCBD-EGF)	*E. coli*	Skin wounds, catheter-injured arteries, and hind limb muscles	[284]
RGD/EGF/hydrophobic sequence E12 (ERE–EGF)	*E. coli*	Controlling cell functions	[285]
NGF-β + collagen binding domain	*E. coli* BL21 strain	Delivery system for neuronal development and regeneration	[286]